Isolation and Identification of Microorganisms in JSC Mars-1 Simulant Soil. Claudia Mendez[1], Elizabeth Garza[1], Poonam Gulati[1], Penny A. Morris[1], and Carlton C. Allen[2], [1]Dept. of Natural Sciences, University of Houston-Downtown, One Main Street, Houston, TX 77002, gulatip@uhd.edu; [2]NASA/Johnson Space Center, Houston, Tx. 77058, carlton.c.allen@nasa.gov

Introduction: Microorganisms were isolated and identified in samples of JSC Mars-1, a Mars simulant soil. JSC Mars-1 is an altered volcanic ash from a cinder cone south of Mauna Kea, Hawaii [1]. This material was chosen because of its similarity to the Martian soil in physical and chemical composition. The soil was obtained by excavating 40 cm deep in a vegetated area to prevent contamination. In previous studies, bacteria from this soil has been isolated by culturing on different types of media, including minimal media, and using biochemical techniques for identification [2]. Isolation by culturing is successful only for a small percentage of the population. As a result, molecular techniques are being employed to identify microorganisms directly from the soil without culturing [3]. In this study, bacteria were identified by purifying and sequencing the DNA encoding the 16s ribosomal RNA (16s rDNA). This gene is well-conserved in species and demonstrates species-specificity. In addition, biofilm formation, an indicator of microbial life, was studied with this soil. Biofilms are microbial communities consisting of microbes and exopolysaccharides secreted by them. This is a protective way of life for the microbes as they are more resistant to environmental pressures [4].

Materials: Bacteria were cultured in 1/10 strength Miller's Luria Broth (LB) (Bector-Dickinson (BD)). DNA from soil was purified using the UltraClean Soil DNA Kit and the UltraClean Mega Prep Soil DNA Kit (for 10 gram soil samples). An UltraClean Microbial DNA Isolation Kit was used to isolate DNA directly from bacteria (all three kits from MO BIO Laboratories, Inc.). The DNA was amplified and cloned using the TOPO TA Cloning Kit For Sequencing with pCR 4-TOPO Vector, and the TOPO TA Cloning Kit pCR 2.1-TOPO Vector, (both kits from Invitrogen Life Technologies). Techgene Thermal Cycler PCR machine (Techne) was used to amplify DNA. The cloned DNA was transformed into *E.coli* cells using the One Shot TOP 10 Chemically Competent Cells (Invitrogen). The DNA was prepared from the transformed cells using the QIAprep Spin Miniprep Kit (QIAprep).

For biofilm formation, sterile 24 well, flat-bottom plates with lids (Costar) were used. Biofilms were stained with crystal violet (CV), that adheres to them. A measurement of CV absorbance at 600nm is proportional to the amount of biofilm on the plate wells. The absorbance was measured using a UV-1601 UV-Visible Spectrophotometer attached to a CPS-240A cell positioner and a CPS Controller (Shimadzu) [5].

Soil DNA Extraction: DNA was extracted from 0.5 grams or from 10 grams of JCS Mars-1 simulant soil, using the DNA extraction protocols in the kits. Due to low yields, DNA was also extracted after overnight culture of the soil in 1/10 LB. The soil and the liquid part of the cultures were separated. The soil was used to extract DNA using the UltraClean Soil DNA Kit and the liquid from the culture tubes was used to extract DNA using the UltraClean Microbial DNA Isolation Kit. In some cases, DNA from the soil was too dilute and was concentrated using glycogen (2µl of a 20µg/µl solution in a 100µl sample), 1/10 volume of 5M potassium acetate (KoAc) and 2X volume of 100% ethanol. Following an overnight incubation at -40°C, the solution was centrifuged, washed with 70% ethanol, air-dried, the pellet resuspended in 50µl of deionized water and stored at -20°C.

Polymerase Chain Reaction for DNA Amplification: All of the purified DNA samples were used in a PCR with Universal Forward and Bacterial Reverse primers. A 1.5% agarose gel was done in order to note the presence of a1500 base pair band.

Transformation of E. coli Cells: After the PCR, the DNA was ligated to a plasmid vector and transformed into competent *E.coli* cells as described in the protocol from TOPO TA Cloning kit. The transformants were checked for the presence of the DNA by extracting the plasmid DNA using the QIAprep Spin Miniprep Kit. The plasmid DNA was digested with the restriction enzyme *EcoRI* and the presence of the 1500 base pair was confirmed by agarose gel electrophoresis.

Biofilm formation: Biofilms were allowed to form in sterile 24 well cell culture plates. Three wells with the JSC Mars-1 simulant soil in 1/10 LB and 3 wells with 1/10 LB only were set up. After incubating at room temperature for 3 days with shaking, crystal violet (CV) was added and the amount of stain that adhered to the biofilm was analyzed by measuring the absorbance at 600nm in a spectrophotometer.

Results: *Identification of Bacteria*: In this study, analysis of 16s rDNA was used to identify some bacteria in the JSC Mars-1 simulant soil. As seen in Table 1, the most frequent bacterial genus identified was *Bacillus*. This result is not unexpected because *Bacillus* is a soil organism and lives on vegetated soil horizons like the area from which the JSC Mars-1 simulant soil was obtained. The same is true for the genus *Clostridium*. On the other hand, some new organisms were identified such as *Eubacterium tenue*, *Tepidibacter formicigenes* and swine manure bacterium.

Table 1. Bacteria Identified in JSC Mars-1

Organism	Number of repetitions
Clostridium sordelli	0
Bacillus firmis	0
Bacillus luciferensis	2
Eubacterium tenue	1
Bacillus drentensis	0
Clostridium subterminale	1
Bacillus cereus	0
Bacillus silvestris	1
Bacillus macroides	0
Bacillus weihenstephanensis	0
Tepidibacter formicigenes	0
Bacillus bataviensis	1
Bacillus simplex	0
Swine manure bacterium	0

Measurement of Biofilms: Biofilm formation in 24-well plates was measured using a crystal violet assay. Crystal violet stain adheres to the biofilm and is a measure of the quantity of biofilm on the surface. The higher the absorbance value of the crystal violet, the more the biofilm. As seen from Table 2, the JSC Mars-1 simulant soil samples have higher values than the media control samples.

Table 2. Absorbance Values for Crystal Violet

Sample	Absorbance at 600nm
Well A1- JSC Mars-1 Soil	0.370
Well B1- JSC Mars-1 Soil	0.238
Well C1- JSC Mars-1 Soil	0.219
Well A6- Media Control	0.103
Well B6- Media Control	0.141
Well C6- Media Control	0.113

In this study, JSC Mars-1 was assayed for the presence of bacteria. Molecular analysis of the soil revealed several species of bacteria. Since this technique does not rely on culturing of bacteria, it does not favor the identification of only those organisms that are culturable. Further efforts will identify other bacteria, Archea and fungi. Biofilm formation was also studied with this soil, and it was demonstrated that biofilms do form on solid surfaces. The formation of biofilms is an indicator of microbial presence in the soil. These studies reveal that there is microbial life in the JSC Mars-1 simulant soil.

References: [1] Allen, C. C. et al. (1998) *Lunar and Planetary Science XXIX.* [2] Allen, C. C. et al. (2000) *Lunar and Planetary Science XXXI.* [3] Holmes, J. et al. (2002) *Annual Reviews of Microbiology 56*, p. 212-243. [4] Stoodley, P. et al. *Annual Reviews of Microbiology 56*, p. 174-211. [5] “Colorimetric Measurement of Biofilm Density”, biofilmsonline.com

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